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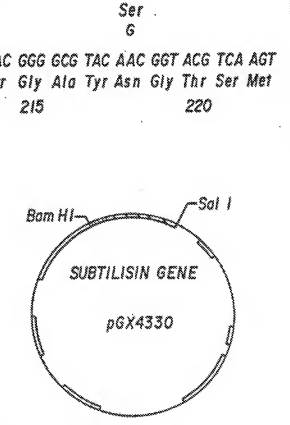
(57) Abstract

Cloned DNA is mutated by creating a single-stranded target region in a cloned DNA segment, and introducing a mutation into the single-stranded target region by treating the target region with a chemical or biological mutagenizing agent capable of introducing mutations into single-stranded DNA. The mutated target region then is rendered double-stranded and a microorganism is transformed with the mutated double-stranded DNA present in an expression vector. The transformed microorganism is cultivated under conditions wherein the mutated DNA is expressed to form an expression product, and the expression product is screened to identify a desired mutation in the DNA segment. Mutant subtilisins of enhanced thermal stability are also disclosed.



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MUTAGENESIS AND SCREENING METHOD AND PRODUCT

BACKGROUND OF THE INVENTION

Cross-Reference to Related Application

This is a continuation-in-part of application Serial No. 828,545, filed in the U.S. on February 12, 1986, the contents of which are fully incorporated herein by reference.

Field of the Invention

The present invention relates to a method for mutating genetic material and screening mutant genetic material for a desired mutation. The invention further relates to mutant genetic material produced according to the disclosed method, as well as to expression products of such mutant genetic material.

Description of the Background Art

The largest class of naturally occurring proteins is made up of enzymes. Each enzyme generally catalyzes a different kind of chemical reaction, and is usually highly specific in its function. An enzyme molecule contains an active site to which a specific substrate is bound during a catalytic cycle.

Although there may be slight variations in a distinct type of naturally occurring enzyme within a given species of organism, enzyme molecules of a specific type produced by organisms of the same species generally are substantially identical with respect to substrate specificity, thermal stability, activity levels under various conditions (e.g., temperature and pH), oxidation stability, and the like. Such characteristics of a naturally occurring or "wild-type" enzyme are not necessarily optimized for utilization outside of the natural environment of the enzyme. It may thus be desirable to alter a natural characteristic of an enzyme to optimize a certain property of the enzyme for a specific use, or for use in a specific environment.

The amino acid sequence of an enzyme determines the characteristics of the enzyme, and the enzyme's amino acid sequence is specified by the nucleotide sequence of a gene coding for the enzyme. A change of the amino acid sequence of an enzyme may alter the enzyme's properties to varying degrees, or may even inactivate the enzyme, depending on the location, nature and/or magnitude of the change in the amino acid sequence.

Methods for introducing specific amino acid changes into proteins, such as oligonucleotide-directed mutagenesis of DNA, are known in the art. However, the ability to predict the effects of a specific mutation is poor, making the process of creating and characterizing desired mutations laborious.

Methods for random mutagenesis of genetic material are also known, but in the absence of a method for rapid and effective screening of a large

number of mutants, identification of organisms having the desired mutation is tedious.

There remains a need in the art for new and practical mutagenesis and screening methods and for the products thereof.

Naturally occurring bacterial proteases are currently used for many purposes, among these is as an additive to washing preparations. Many stains on clothes are proteinaceous and wide-specificity proteases can substantially improve removal of such stains. Unfortunately, naturally-occurring proteases lose activity when stored in solution with detergents. Typically this decay of activity is geometric in nature, that is, a certain percentage of activity is lost in each time interval. The present invention provides a method to develop novel proteases with enhanced thermal stability and which survive prolonged storage in liquid detergents much longer than naturally-occurring proteases.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method for mutating cloned DNA and thereafter identifying mutants comprises creating a single-stranded target region in a cloned DNA segment, and introducing a mutation into the target region by treating the target region with a chemical mutagenizing agent capable of introducing mutations into single-stranded DNA. The target region then is rendered double-stranded to form mutated double-stranded DNA, and a microorganism is transformed with an expression vector containing the mutated

double-stranded DNA. The transformed microorganism is cultivated under conditions wherein the mutated DNA is expressed to form an expression product, and the expression product is screened to identify a desired mutation in the DNA segment.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram showing the subtilisin gene cloned in plasmid pGX4330 for mutagenesis and screening according to the invention.

FIG. 2 is a schematic diagram showing a gapped duplex DNA molecule for mutagenesis according to the invention.

FIG. 3 is a portion of the variant subtilisin DNA sequence showing the single base substitution in the subtilisin gene which produces subtilisin of enhanced thermal stability according to the invention.

FIG. 4 is a graphic illustration showing thermal inactivation of variant subtilisin according to the invention (7150) and subtilisin wild-type at 65°C in 10 mM $CaCl_2$, 50 mM KCl, 50 mM Tris-HCl, pH 8.0.

FIG. 5 is a graphic illustration showing thermal inactivation of variant subtilisin according to the invention (7150) and subtilisin wild-type at 45°C in 1.0 mM EDTA, 50 mM KCl, 50 mM Tris-HCl, pH 8.0.

FIG. 6 is a graphic illustration showing thermal inactivation of variant subtilisin according to the invention (7150) and subtilisin wild-

type at 40°C, in 1.0 mM EDTA, 20 mM 3-(cyclohexyl-amino) propanesulphonic acid (CAPS), pH 10.5.

FIG. 7 is a graphic illustration showing differential scanning calorimetry (DSC) profiles for variant subtilisin according to the invention (7150) and subtilisin wild-type at a protein concentration of 3.0 mg/ml (scan rate 60°C/hr.). Samples were scanned in 50 mM Tris-HCl, pH 8.0, 50 mM KCl with either 10mM EDTA or 10mM $CaCl_2$.

FIG. 8 is a graphic illustration showing thermal stability of variant subtilisins according to the invention and subtilisin wild-type at 70°C in 10 mM $CaCl_2$, 50 mM Tris- HCl, 50 mM NaCl at pH 8.0.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to modification of one or more characteristics of a protein, such as an enzyme, by mutating a nucleotide sequence (gene) coding for the protein and screening for desired mutations. Such modification can include enhancing or diminishing an enzyme's thermal stability, substrate specificity, oxidation stability, activity profile under varying conditions of pH and/or temperature, and the like.

Prior to mutation of a gene coding for an enzyme of interest, the gene generally is first isolated from its natural source and cloned in a cloning vector. Alternatively, mRNA which is transcribed from the gene of interest can be isolated from the source cell and converted into cDNA by reverse transcription for insertion into a cloning vector. A cloning vector can be a phage or

plasmid, and generally includes a replicon for autonomous replication of the vector in a microorganism independent of the genome of the microorganism. A cloning vector advantageously includes one or more phenotypic markers, such as DNA coding for antibiotic resistance, to aid in selection of microorganisms transformed by the vector.

Procedures for insertion of DNA or cDNA into a vector for cloning purposes are well known in the art. These procedures generally include insertion of the gene of interest into an opened restriction endonuclease site in the vector, and may involve addition of homopolymeric tails of deoxynucleotides to the ends of the gene and linking the gene to opened ends of a cloning vector having complementary homopolymeric tails. A gene of interest present in a cloning vector can be mutated in accordance with the method of this invention.

In one embodiment, a gene of interest to be treated according to the invention is present in an expression vector. An expression vector generally falls under the definition of a cloning vector since an expression vector usually includes the components of a typical cloning vector, namely, one or more replicons as defined above, and one or more phenotypic markers for selection purposes. Additionally, an expression vector includes control sequences encoding a promoter, operator, ribosome binding site and translation initiation signal. For expression under the direction of the control sequences, a target gene to be treated according to the invention is operably linked with the control sequences in the proper reading frame.

An expression vector containing the DNA sequence to be targeted can be a phage or a plasmid, with plasmids being preferred.

According to one aspect of the invention, a method for mutating a cloned DNA segment and thereafter identifying mutants includes the step of providing a single-stranded target region in the cloned DNA segment. The target region can include the entire DNA segment of interest, or a portion thereof. The targeted portion can be randomly or specifically selected.

The target region of the DNA segment of interest is rendered single-stranded by any suitable method known in the art, such as the method disclosed in Shortle et al., Proc. Natl. Acad. Sci. USA, 75:2170-2174 (1978). Another method involves providing a single-stranded copy of a plasmid containing the DNA segment of interest, and annealing to the single-stranded copy a DNA fragment containing plasmid sequences but not including a segment complementary to the target region. This procedure creates a gapped duplex molecule with a single-stranded target region.

One or more mutations are introduced into the single-stranded target region by treating the target region with a chemical or biological mutagenizing agent (mutagen) capable of introducing mutations into single-stranded DNA. One such chemical mutagen is sodium bisulfite which causes G-C to A-T transitions only (wherein G, C, A or T refer respectively to guanine, cytosine, adenine and thymine). Other chemical or biological mutagens, such as hydroxylamine, nitrous acid,

formic acid and hydrazine are suitable and can be used according to this invention.

Mutations are introduced at a controllable level, advantageously from one to about five changes per molecule, and a library of mutated plasmid-borne DNA is produced (e.g., by incubating DNA in 4M Na-bisulfite, pH 6.0 for 5 to 30 minutes). Advantageously, the library is large enough to generate up to 10^5-10^6 different variants upon transformation into a microorganism, since a desired mutation may occur infrequently.

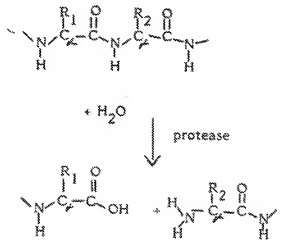
Alternatively, mutations are introduced into the cloned DNA by replication of the cloned DNA in a mutator strain of E. coli, such as a Mut D strain of E. coli which produces a range of mutations due to its error prone DNA polymerase. In this method Mut D E. coli containing plasmid are grown at 37°C in 250 ml culture in rich media containing 50 mg/ml thymine to an optical density at 650 nm of 0.6. The plasmid is amplified by the addition of 175 ug/ml chloramphenicol and continued incubation for 16 hours at 37°C.

After mutating the target region, the target region is rendered double-stranded by filling in the single-stranded region using DNA polymerase I (Klenow fragment) or reverse transcriptase, see, e.g., Shortle et al., supra.

A microorganism is transformed with the mutated double-stranded DNA present in an expression vector, the mutated DNA being operably linked to control sequences capable of directing expression of the mutated DNA in the transformed microorganism. The transformed microorganism then is cultivated under protein-producing conditions

including necessary nutrients and physiologically acceptable pH and temperature, such that the mutated DNA is expressed to form an expression product.

The method according to this invention can be used to mutate serine proteases to enhance certain characteristics, particularly thermal stability. A protease is a catalyst for the cleavage of peptide bonds.



A serine protease is an enzyme which catalyzes the hydrolysis of peptide bonds in which there is an essential serine residue at the active site. Serine proteases can be inhibited by phenylmethanesulfonylfluoride and by diisopropylfluorophosphate. A subtilisin is a serine protease produced by Gram positive bacteria or fungi. The amino acid sequences of seven subtilisins are known. These include five subtilisins from Bacillus strains (subtilisin BPN', subtilisin Carlsberg, subtilisin

DY, subtilisin amylosacchariticus, and mesenticopeptidase). (Vasantha et al., "Gene for alkaline protease and neutral protease from Bacillus amyloliquefaciens contain a large open-reading frame between the regions coding for signal sequence and mature protein," J. Bacteriol. 159:811-819 (1984); Jacobs et al., "Cloning sequencing and expression of subtilisin Carlsberg from Bacillus licheniformis," Nucleic Acids Res. 13:8913-8926 (1985); Nedkov et al., "Determination of the complete amino acid sequence of subtilisin DY and its comparison with the primary structures of the subtilisin BPN', Carlsberg and amylosacchariticus," Biol. Chem. Hoppe-Seyler 366:421-430 (1985); Kurihara et al., "Subtilisin amylosacchariticus," J. Biol. Chem. 247:5619-5631 (1972); and Svendsen et al., "Complete amino acid sequence of alkaline mesentericopeptidase," FEBS Lett. 196:228-232 (1986)).

The amino acid sequence of the subtilisin thermitase from Thermoactinomyces vulgaris is also known. (Meloun et al., "Complete primary structure of thermitase from thermoactinomyces vulgaris and its structural features related to the subtilisin-type proteanases," FEBS Lett. 183:195-200 (1985).)

The amino acid sequences from two fungal proteases are known: proteinase K from Tritirachium album (Jany et al., "Proteinase K from Tritirachium albam Limber," Biol. Chem. Hoppe-Seyler 366:485-492 (1985)) and thermomycolase from the thermophilic fungus, Malbranchea pulchella (Gaucher et al., "Endopeptidases: Thermomycolin," Methods Enzymol. 45:415-433 (1976)).

These enzymes have been shown to be related to subtilisin BPN', not only through their primary

sequences and enzymological properties, but also by comparison of x-ray crystallographic data. (McPhalen et al., "Crystal and molecular structure of the inhibitor eglin from leeches in complex with subtilisin Carlsberg," FEBS Lett. 188:55-58 (1985) and Pahler et al., "Three-dimensional structure of fungal proteinase K reveals similarity to bacterial subtilisin," EMBO J. 3:1311-1314 (1984).)

As used in this invention, the term "subtilisin material" is a proteinaceous material which contains a subtilisin as its active ingredient. As used herein, and under the definition of subtilisin material, any serine protease is a subtilisin which has at least 30%, preferaby 50%, and more preferably 80% amino acid sequence homology with the sequences referenced above for subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin amylosacchariticus, mesenticopeptidase, thermitase, proteinase K, and thermomycolase. These serine proteases are also described herein as "homologous serine proteases."

According to one embodiment, B. subtilis is transformed by an expression vector carrying the mutated DNA. If expression is to take place in a secreting microorganism such as B. subtilis, a single sequence may follow the translation initiation signal and precede the DNA sequence of interest. The signal sequence acts to transport the expression product to the cell wall where it is cleaved from the product upon secretion. The term "control sequences" as defined above is intended to include a signal sequence, when it is present.

For screening mutants, transformed B. subtilis is cultivated in the presence of a filter material

(such as nitrocellulose) to which the secreted expression product (e.g., enzyme) binds. In order to screen for an expression product having a desired characteristic, filter bound expression product is subjected to conditions which distinguish expression product of interest from wild-type expression product. For example, the filter-bound expression product can be subjected to conditions which would inactivate a wild-type expression product. The treated expression product can thereafter be contacted with a substrate of the enzyme, and enzyme activity with the substrate identifies an expression product with enhanced stability and thereby a desired mutation.

Although the invention will further be specifically described with respect to production of thermally stable variants of the Bacillus serine protease, subtilisin BPN', it is to be understood that the present invention is equally applicable to modification of the characteristics of other proteins. In particular, other homologous serine proteases from other microorganisms may be mutated and screened according to this invention. These homologous serine proteases may include, but are not limited to, those from other Bacillus strains such as subtilisin Carlsberg from Bacillus licheniformis, subtilisin DY, subtilisin amylosacchariticus, and mesentericopeptidase. Fungal proteases, such as protease K and thermomycolase may also be used, as well as mammalian proteases produced in a bacterial host.

For mutating subtilisin genetic coding sequences and isolating variant subtilisin proteins of enhanced thermal stability, the subtilisin gene

from a Bacillus species including the natural promoter and other control sequences is cloned into a plasmid vector containing replicons for both E. coli and B. subtilis, a selectable phenotypic marker, and the M13 origin of replication for production of single-stranded plasmid DNA upon superinfection with helper phage IR1. Single-stranded plasmid DNA containing the cloned subtilisin gene is isolated and annealed with a DNA fragment containing vector sequences but not the coding region of subtilisin, to create a gapped duplex molecule. Mutations are introduced into the subtilisin gene either with sodium bisulfite, nitrous acid, or formic acid or by replication in a mutator strain of E. coli as described above. Since sodium bisulfite reacts exclusively with cytosine in single-stranded DNA, the mutations created with this mutagen are restricted only to the coding regions. Reaction time and bisulfite concentration are varied in different experiments such that from one to five mutations are created per subtilisin gene on average. Incubation of 10 ug of gapped duplex DNA in 4 M Na-bisulfite, pH 6.0, for 8 minutes at 37°C in a reaction volume of 400 ul, deaminates about 1% of cytosines in the single-stranded region. The coding region of mature subtilisin contains about 200 cytosines, depending on the DNA strand. Advantageously, the reaction time is varied from about 4 minutes (to produce a mutation frequency of about one in 200) to about 20 minutes (about 5 in 200).

After mutagenesis, the gapped molecules are treated in vitro with DNA polymerase I (Klenow fragment) to make fully double-stranded molecules

and to fix the mutations. Competent E. coli are then transformed with the mutagenized DNA to produce an amplified library of mutant subtilisins. Amplified mutant libraries can also be made by growing the plasmid DNA in a Mut D strain of E. coli which increases the range of mutations due to its error prone DNA polymerase.

The mutagens, nitrous acid and formic acid may also be used to produce mutant libraries. Because these chemicals are not as specific for single-stranded DNA as sodium bisulfite, the mutagenesis reactions are performed according to the following procedure. The coding portion of the subtilisin gene is cloned into M13 phage by standard methods and single-stranded phage DNA prepared. The single-stranded DNA is then reacted with 1M nitrous acid pH 4.3 for 15-60 minutes at 23°C or 2.4M formic acid for 1-5 minutes at 23°C. These ranges of reaction times produce a mutation frequency of from 1 in 1000 to 5 in 1000. After mutagenesis, a universal primer is annealed to the M13 DNA and duplex DNA is synthesized using the mutagenized single-stranded DNA as a template so that the coding portion of the subtilisin gene becomes fully double-stranded. As this point the coding region can be cut out of the M13 vector with restriction enzymes and ligated into an unmutagenized expression vector so that mutations occur only in the restriction fragment. (Myers et al., Science 229:242-247 (1985)).

To screen for thermostability, a library of subtilisin variants should be produced which is large enough to generate approximately 5×10^4 random subtilisin variants or more upon transforma-

tion into B. subtilis, since mutations resulting in enhanced thermal stability occur infrequently.

After mutagenesis, the variant library is used to transform B. subtilis which will both express and secrete subtilisin. The subtilisin-bearing plasmid advantageously contains a high copy B. subtilis replicon and is capable of producing subtilisin at a high level in a Bacillus host. To screen for stable variants, a protease deficient B. subtilis strain is transformed with the variant plasmid library and plated out as follows: A nitrocellulose filter is placed on a nutrient base in a petri dish, and a cellulose acetate filter is placed on top of the nitrocellulose. Colonies are grown on the cellulose acetate, and subtilisin from individual colonies is secreted through the cellulose acetate onto the nitrocellulose filter where it is stably bound. Subtilisin from hundreds of colonies is bound to a single filter allowing subsequent screening of thousands of different variants by processing multiple filters.

To identify colonies producing subtilisin of enhanced thermal stability, the filters are incubated at 70°C for 30 minutes in buffer solution to inactivate substantially all wild-type subtilisin activity. Variant subtilisins of enhanced stability retain activity after this heating step. When stable variants have been further mutagenized to screen for additional increases in stability, higher temperatures of longer incubation times must be used to inactivate the background activity. The heat-treated filter then is soaked in a solution containing Tosyl-L-Arg methyl ester (TAME) (Sigma) and the pH indicator phenol red (Kodak). Because

TAME is a substrate for subtilisin it is cleaved in zones on the filter containing variant subtilisins which remain active after thermal treatment. As cleavage occurs, protons are released in the reaction and cause phenol red to change in color from red to yellow in areas retaining protease activity.

This procedure can be used to screen for other classes of variants with only slight modifications. For example, the filters could be treated at high pH, with denaturants, oxidizing agents, or under other conditions which normally inactivate an enzyme such as subtilisin, to find resistant variants. Variants with altered substrate specificity could be screened by replacing TAME with other substrates which are normally not cleaved by wild-type subtilisin.

Once a variant of enhanced stability is identified by screening, the colony from which the variant is derived is isolated and the altered subtilisin is purified. Experiments can be performed on the purified enzyme to determine conditions of thermal inactivation, denaturation temperature, kinetic parameters as well as other physical measurements. The altered gene can also be sequenced to determine the amino acid changes responsible for the enhanced stability. Using this procedure, variants showing a one-and-one-half to eight-fold improvement in resistance to thermal inactivation at 65°C have been isolated. Thermally stable subtilisin is useful in laundry cleaning compositions.

It has surprisingly been found that substituting another amino acid for asparagine at amino acid

position 218 of subtilisin enhances the thermal stability of subtilisin. Without being bound to any particular theory, it is believed that substitution of serine for asparagine at position 218 stabilizes subtilisin by increasing the change in free energy for the unfolding reaction. According to one embodiment of this invention, the present invention relates to a cloned mutant subtilisin gene coding for a subtilisin material with serine substituted for asparagine at amino acid position 218 of subtilisin. This variant, produced by strain GX7150, has been designated 7150. This invention also includes other amino acid substitutions at the amino acid position 218 of subtilisin, especially aspartic acid.

In another embodiment of this invention, the gene coding for a subtilisin material contains serine or aspartic acid at amino acid position 218 and the subtilisin gene may also contain one or more additional amino acid substitions. Among the preferred additional amino acid substitutions in the cloned mutant 218-substituted subtilisin gene are the following random mutated subtilisin variants produced by the indicated strains:

STRAIN	AMINO ACID SUBSTITUTION(S)	VARIANT
GX7169	ASN218 ---> SER GLY131 ---> ASP	7169
GX8301	ASN218 ---> SER THR254 ---> ALA	8301
GX8306	ASN218 ---> SER GLY166 ---> SER	8306
GX8315	ASN218 ---> SER GLY131 ---> ASP THR254 ---> ALA	8315

Other substitutions which increase the thermal stability of subtilisin are:

STRAIN	SUBSTITUTION(S)	VARIANT
GX7142	ALA116 ---> THR GLY131 ---> ASP	7142
GX7148	GLY131 ---> ASP	7148
GX7178	SER188 ---> PRO	7178
GX7188	ALA116 ---> GLU	7188
GX7189	LEU126 ---> ILE	7189
GX8305	SER53 ---> THR	8305

All of the amino acid substitutions given above were produced by random mutagenesis and screened according to this invention. Once the random mutations are made and identified, the mutation can be repeated and reproduced by oligonucleotide-directed mutagenesis. Furthermore, when more than one stabilizing mutation is introduced into the subtilisin molecule, the effects of those changes appear to be additive in free energy.

In addition to the subtilisin gene, genes for other serine proteases or other types of enzymes

may be mutagenized to produce products with enhanced characteristics.

In addition to applying the method as described to other serine proteases, one can use the information obtained from one serine protease, subtilisin BPN' for example, to improve other proteases which are closely related, subtilisin Carlsberg for example. Closeness of relation is measured by comparison of amino-acid sequences. There are many methods of aligning protein sequences, but the differences are only manifest when the degree of relatedness is quite small. The methods described in Atlas of Protein Sequence and Structure, Margaret O. Dayhoff editor, Vol. 5 Supplement 2, 1976, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, DC., p. 3 ff., entitled SEARCH and ALIGN, define relatedness. As is well known in the art, related proteins can differ in number of amino acids as well as identity of each amino acid along the chain. That is, there can be deletions or insertions when two structures are aligned for maximum identity. For example, subtilisin Carlsberg has only 274 amino acids, while subtilisin BPN' has 275 amino acids. Aligning the two sequences shows that Carlsberg has no residue corresponding to ASN56 of subtilisin BPN'. Thus the amino acid sequence of Carlsberg would appear very different from BPN' unless a gap is recorded at location 56. Therefore, one can predict with high degree of confidence that substituting SER for ASN at location 218 of subtilisin Carlsberg will increase thermal stability, provided that the

residues in Carlsberg are numbered by homology to BPN'.

When one of the two homologous subtilisins has a gap, one must infer that the structures are different at that locality. Examples of such differences are well known in the art. Because of these local differences, one should not transfer stabilizing mutations if either subtilisin has a gap at, or immediately adjacent, to the site of the mutation. Therefore, after aligning the amino acid sequences, those mutations at or next to gaps are deleted from the list of desirable mutations and the mutation is not made.

One can use this reasoning to transfer all of the randomly-obtained stabilizing mutations described herein to other homologous serine proteases.

The stabilizing mutations found by the random mutagenesis method reveal that the structure of the enzyme being studied is not optimal at that location. The random method usually changes very few bases within the gene. Usually only one base is changed within a given codon; thus, one cannot go from any one amino acid to all other amino acids. For example, the codons for ASP (GAT and GAC) can be changed by single-base changes to (CAT, TAT, AAT, CAC, TAC, AAC, GGT, GTT, GCT, GGC, GTC, GCC, GAA, or GAG) which code for HIS, TYR, ASN, GLY, ALA, VAL, or GLU, but not to PHE, LEU, ILE, MET, THR, SER, CYS, LYS, ARG, GLN, TRP, or PRO.

Once the random method has shown that a given site is not optimal, oligonucleotide-directed mutagenesis can be used to introduce each of the

remaining 18 amino acids at that site. These mutants can then be tested for improved properties.

The mutant subtilisin material of this invention can be used as an additive to washing preparations, such as detergents, which are used for cleaning, in particular for cleaning clothes. The mutant subtilisin material of this invention is more thermally stable than wild-type subtilisin material and thus does not lose activity as rapidly as wild-type when stored in solution with detergents or when subjected to high heat during use in cleaning. By use of the mutant subtilisin material of this invention as an additive in washing preparations, the removal of proteinaceous stains on fabric is improved. The amount of mutant subtilisin material that may be used as an additive to washing preparations are well known in the art, or may readily be ascertained by routine experimentation. The optimal range of enzyme concentration will, of course, be related to the cost of the enzyme and the amount of cleaning needed.

The invention is illustrated by the following examples which are not intended to be limiting.

EXAMPLE I

Mutagenesis of Subtilisin

For mutagenesis, the subtilisin gene from Bacillus amyloliquefaciens including the natural promoter sequences was isolated (Vasantha et al. (1984) J. Bacteriology 159:811-819) and cloned into a vector (pGX4330, Figure 1) containing the beta-lactamase gene and replicon from pBR322 for growth in E. coli; the kanamycin nucleotidyl transferase

gene and replicon from pUB110 for growth in B. subtilis; and the M13 origin of replication for production of single-stranded plasmid DNA upon superinfection with helper phage IR1. Mutations were introduced into the subtilisin gene either with sodium bisulfite or by replication in a mutator strain of E. coli. Sodium bisulfite mutations were restricted to the subtilisin coding region using a variation of the method of Folk and Hofstetter (1985) Cell 33:585-593). Single-stranded plasmid DNA was annealed with a DNA fragment containing vector sequences but not the coding region of subtilisin to create a gapped duplex molecule (Figure 2). One ug of single-stranded pGX4330 was mixed with 1 ug of double-stranded pGX4330 cut with BamHI and SalI in 50 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$ in a volume of 15 ul. The DNA was heated to 90°C in a boiling water bath for 5 minutes and allowed to anneal at 60°C for 10 minutes. The gapped duplex was reacted with sodium bisulfite pH 6.0 in a volume of 400 ul at 37°C. Reaction time was varied from four to twenty minutes to produce an average of one to five mutations per gene. Since sodium bisulfite reacts exclusively with cytosine in single-stranded DNA, the mutations were restricted only to the subtilisin coding region. After mutagenesis, the gapped molecules were treated in vitro with DNA polymerase I (Klenow fragment) using well known techniques (see, e.g., Folk and Hofstetter, supra) to make fully double-stranded molecules and to fix the mutations. Competent E. coli then were transformed with the mutagenized DNA using standard procedures (see, e.g., Maniatis et

al. (1983) "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Press) to produce an amplified library of mutant subtilisins. Libraries were also made by growing pGX4330 in a Mut D strain of E. coli which produces a wider range of mutations due to its error prone DNA polymerase.

EXAMPLE II

Expression and Secretion in B. subtilis and Screening for Altered Stability

The variant library produced in Example I was used to transform B. subtilis strain GX4935, which will both express and secrete subtilisin. The subtilisin bearing plasmid contains a high copy B. subtilis replicon and is capable of producing subtilisin at a high level in a Bacillus host. To screen for stable variants, apr$^-$, npr$^-$, B. subtilis strain GX4935 was transformed with the variant plasmid library and plated out as follows. Two filters were placed on Tryptose Blood Agar Base plus 10 ug/ml kanamycin plates. A nitrocellulose filter was laid directly on the agar and a cellulose acetate filter was placed on top of it. Colonies grown on the top filter secreted subtilisin through the permeable cellulose acetate onto the nitrocellulose where it was stably bound. Subtilisin from hundreds of colonies was bound to a single filter allowing subsequent screening of thousands of different variants by processing multiple filters.

Filters with subtilisin bound thereto were incubated at 70°C in 10 mM $CaCl_2$, 10 mM Tris-HCl pH 8.0 for 30 minutes which inactivates substantially

all native or wild-type subtilisin activity. Variant proteases of enhanced stability retain activity after the heating step.

To detect filter-bound protease activity, the filters were soaked in a solution containing Tosyl-L-Arg methyl ester (TAME) (10mM) and the pH indicator phenol red (1mM) titrated to pH 9.0. TAME is a good substrate for subtilisin and is cleaved in zones on the filter containing active subtilisin. During the procedure, as cleavage occurred, protons were released in the reaction, causing phenol red to change in color from red to yellow in areas of protease activity. This assay procedure distinguishes thermally stable variants.

EXAMPLE III

Primary Characterization of Stable Variants

Once candidates were identified by filter screening, the corresponding colonies from the cellulose acetate filter were grown in liquid culture (2% yeast extract, 10 ug/ml kanamycin) for 18 hours. Under these conditions, subtilisin was secreted into the culture broth at 0.5-1.0 mg/ml and represents > 80% of the total extracellular protein detectable by SDS polyacrylamide gel electrophoresis. The variant subtilisins were tested for resistance to thermal inactivation in solution. Culture supernatant was diluted 20-fold into either 10 mM $CaCl_2$, 50 mM Tris-HCl pH 8.0 or 5mM EDTA 50 mM Tris-HCl pH 8.0. In the presence of calcium, which greatly stabilizes subtilisin, samples were incubated at 65°C and at time intervals up to 90 minutes, aliquots were removed and activity was measured at 37°C using Azocoll as a

substrate. In the presence of EDTA timepoints were taken after incubation at 45°C. Variants whose half-time of thermal inactivations was greater than 150% of wild-type subtilisin were of interest and further characterized. To confirm that stable subtilisin phenotypes resulted from plasmid-borne mutations, plasmid from positive colonies was purified and used to retransform B. subtilis. In almost all cases, the retransformed B. subtilis behave as the original isolate. To sequence the stable variant genes, the plasmid was used to transform E. coli and single-stranded template was made by superinfecting with phage IR1 according to the procedures described in (Dente et al. (1983) Nucleic Acids Res., 11:1645-1655). One variant (GX7150, deposited with American Type Culture Collection, Rockville, Maryland as ATCC No. 53459) that produces a thermally stable subtilisin was shown to have a single base change in the cloned subtilisin gene resulting in a substitution of asparagine 218 with serine (see Fig. 3). To ensure that the enhanced stability resulted solely from this change, the mutation was introduced into wild-type subtilisin by oligonucleotide-directed mutagenesis using standard procedures (see, e.g., Zoller and Smith (1983), Methods in Enzymology, 100:468-500). The resulting subtilisin variant was shown to behave exactly like random isolate variant subtilisin produced by GX7150.

EXAMPLE IV

Physical and Chemical Properties of Subtilisin Produced by B. Subtilis Strain GX7150

Purification:

Subtilisin produced by GX7150 as described above, where asparagine 218 has been changed to serine was purified from cell-free fermentation broths by means of the following three-step purification scheme:

(1) DEAE chromatography of crude fermentation broth. The broth was adjusted to pH 7.0 by addition of solid 2-(N-morpholino)ethanesulfonic acid (Mes) and loaded onto a bed (13 x 5 cm) of DE-52 cellulose (Whatman) which was previously equilibrated with 20 mM Mes buffer (pH 7.0). Subtilisin washes through unretarded under these conditions.

(2) Acetone fractionation of DEAE eluate. Acetone (-20°C) was stirred with the DEAE eluate at 4°C. The subtilisin precipitates between 50 and 70% acetone. The fraction the precipitates between 0 and 50% acetone was discarded.

(3) SE-53 (Whatman) chromatography of acetone precipitate. The acetone precipitated subtilisin was dissolved in 20 mM Mes buffer (pH 6.0) and loaded onto a column (2.5 x 16 cm) of SE-53 cellulose equilibrated with the same buffer. A linear salt gradient (0 to 0.2 M NaCl) was used to elute the subtilisin.

Fractions containing the highest specific activities were pooled and stored at -20°C either as 70% isopropanol or 50% ammonium sulfate precipitants.

Enzyme Assay:

Subtilisin activity was assayed by monitoring the hydrolysis of 1.0 mM solutions of the substrate, succinyl(L)-Ala-(L)-Ala-(L)-Pro-(L)-Phe-p-nitroanilide (SAAPF-pNA (Calbiochem)), in 50 mM Tris-HCl (pH 8.0), 50 mM KCl at 25.0°C. One unit of activity corresponds to the amount to enzyme that hydrolyzes 1 umole of substrate per min. under these conditions. One of the products of hydrolysis, p-nitroanilide, has an extinction coefficient of 8800 $M^{-1}cm^{-1}$ at 410 nm, thus allowing easy monitoring of the enzymatic reaction (Delmar et al. (1979) Anal. Biochem., 99:316-320). Subtilisin concentrations were estimated at 280 nm using $E_0(.1\%) = 1.17$ (Ottesen & Svendson (1976) Methods in Enzymology, p. 207).

Kinetics of Thermal Inactivation:

Thermal inactivation studies were performed by incubating a subtilisin solution (0.05 mg/ml) dissolved in a buffer of choice (usually 50 mM Tris-HCl (pH 8.0), 50 mM KCl) at some particular temperature. The sample was placed in a glass Durham tube which was immersed in a thermostated circulated water bath equilibrated at the temperature of choice. Evaporation from the sample tube was prevented by sealing with Parafilm. Aliquots (10 ul) were removed at various time points and assayed by dilution into 1.0 ml of assay solution at 25°C. The time zero measurement was the rate of hydrolysis of SAAPF-pNA before the sample is immersed in the temperature bath. All subsequent

rates of hydrolysis of substrate were measured after immersion in the bath.

Differential Scanning Calorimetry:

Differential scanning calorimetry data was obtained with a Hart Scientific instrument interfaced with an IBM personal computer (model XT) and controlled with DSC Software (Hart Scientific) and the Xenix operating system (Microsoft-Santa Cruz Operations). The temperature was increased from the starting point of 20°C to 90°C at a rate of 60°C/hr. The protein concentration was 3.0 mg/ml.

Isoelectric Focusing:

Analytical electrofocusing of subtilisin in thin layers of polyacrylamide was performed using known techniques, see, e.g. Winter et al. (1977) LKB Application Note 250. Ready-made acrylamide gels, Ampholine PAG plates (pH 3.0 - 9.5) were purchased from LKB, and the calibration standards (pH 5.0 - 10.5) were purchased from Pharmacia. The subtilisin samples were inactivated with phenylmethylsulfonylfluoride (PMSF) before loading onto the gels in order to prevent autolysis during electrofocusing.

Results:

The purified subtilisin 7150 was found to have an isoelectric point of 8.8, essentially indistinguishable from that of the wild-type enzyme. Only a minor contaminant with an isoelectric point of 8.3 was detected. This level of homogeneity is comparable to that of the wild type.

The specific activity of subtilisin 7150 towards a peptide substrate was observed to be 120 ± 5 Units/mg. This is 50% higher than that observed for the wild type: 80 ± 5 Units/mg, an average for more than two dozen separate isolates. This difference is due to small changes in the kinetic parameters for hydrolysis of the substrate rather than a more highly purified preparation.

Thermal Inactivation:

Plots of the logarithm of the remaining activity versus time were found, for the most part, to be linear over the course of three half-lives. Thus, a first order rate law is applicable. This is illustrated in Figure 4 which shows the rate of loss of activity for subtilisin 7150 and the wild-type enzyme at 65°C in the presence of 10 mM $CaCl_2$, 50 mM KCl, and 50 mM Tris-HCl, pH 8.0. Under these conditions, the wild-type enzyme was found to have a half-life of 59 ± 3 minutes which agrees well with that reported in the literature for similar conditions (Voordouw et al. (1976) Biochemistry, 15:3716-3724). The results for subtilisin 7150 reveal that this enzyme has a half-life of 223 ± 16 minutes, almost four times that of the wild type. This is a clear demonstration that the single amino acid change of asparagine 218 to serine dramatically increases the kinetic thermal stability of subtilisin.

The enhanced stability of subtilisin 7150 was further demonstrated for a variety of other conditions. For example, the thermal inactivation of subtilisin 7150 and wild type in the presence of 50 mM KCl and 1.0 mM EDTA, pH 8.0, is shown in

Figure 5. It was necessary to do these experiments at 45°C due to the fact that EDTA negates the known effect of calcium ions in stabilizing subtilisin. Under these conditions, subtilisin 7150 was found to have a half-life almost three times that of the wild-type enzyme.

The apparent greater stability of subtilisin 7150 was also found at high pH (10.5) at 40°C in the presence of 1.0 mM EDTA and protein concentration of 50 ug/ml as shown in Figure 6.

Differential Scanning Calorimetry:

The thermodynamic parameters for the unfolding reaction of subtilisin were obtained through the use of differential scanning calorimetry. The results obtained for subtilisin 7150 and the wild-type enzyme are shown in Figure 7. The unfolding transition for subtilisin 7150 was found to occur at 80.7 ± 0.1°C in 50 mM Tris-HCl pH 8.0, 50 mM KCl, and 10 mM $CaCl_2$ some 2.4° higher than that observed for wild-type under identical conditions. In the presence of 10 mM EDTA, the denaturation temperature of subtilisin 7150 was 62.8°C, about 4°C higher than wild-type subtilisin. These unfolding parameters were obtained by increasing the temperature 60°C/hr. starting at 20°C and finishing at 90°C. The presence of the competitive inhibitor, N-dansyl-3-aminobenzeneboronic acid at a concentration of 2 mM (K_i = 2 um at pH 8.0), greatly reduces the amount of autolysis that accompanies the denaturation process for subtilisin.

The increased temperature of denaturation indicates that the substitution of serine for asparagine at position 218 stabilizes subtilisin by

increasing the change in free energy for the unfolding reaction.

Stability Studies in Liquid Detergent:

For stability studies under alkaline conditions, the subtilisin from B. subtilis strain GX7150 was recovered from a fermentation broth by centrifugation at 7,000 rpm for 30 minutes at 4°C. The subtilisin was then concentrated at 4°C by the addition of ammonium sulfate (495 g/l) and the precipitate collected by centrifugation at 12,000 rpm for 30 minutes at 4°C. The pelleted protein was diluted in deionized water to give a concentration of 40,000 Alkaline Delft Units (ADU)/gm. For comparative purposes, a commercial protease, Enzeco (Enzyme Development, New York, N.Y.) was diluted in deionized water to give a concentration of 40,000 A_{DU}/gm.

The enzymes were incubated at a concentration of 4,000 ADU/gm in the non-phosphate based, U.S. heavy duty liquid detergent Wisk® (a registered trademark of Lever Bros. Co., Inc., N.Y., N.Y.) adjusted to pH 10.0. The solutions were incubated at 25°C for 56 days under these conditions.

Washing tests were performed in a Tergotometer using EMPA-116 (Enzyme Manufacturers Performance Assay from Test Fabrics, Inc., N.Y., N.Y.) as a test fabric. Two grams of Wisk® detergent with enzyme was added to 1 liter of pH 9.0 deionized wash water. Subsequently, three 6" by 6" pieces of EMPA-116 along with three 6" by 6" pieces of EMPA-221 (unsoiled cloth) were added to the wash water. The wash was performed for 15 minutes at 75 RPM agitation at 55°C. After decanting the wash water,

the fabrics were rinsed twice with 1-liter each of cold tap water, dried and lightly ironed. Reflectances of swatches were determined with a Gardner Colorimeter (giving ΔL values). Reflectance was read on both sides of each cloth (a total of 10 readings per cloth). The results are expressed as the mean of 30 ΔL (3 EMPA-116 fabrics) readings for each test. Reflectances were compared to EMPA-116 swatches that were washed, using unmodified subtilisin BPN' and using detergent alone, under identical conditions.

The performance results in the Wisk® detergent as given in Table I.

TABLE I

Sample Tested	Day 1 ΔL	Day 1 % Improve-in Cleaning	Day 35 ΔL	Day 35 % Improve-in Cleaning	Day 56 ΔL	Day 56 % Improve-in Cleaning
Wisk®	26.57	---	28.51	---	26.49	---
Wisk® + GX7150 Modified Subtilisin BPN'	39.05	47	37.60	32	32.99	25
Wisk® + Subtilisin BPN'	37.29	40	34.17	20	26.44	0

EXAMPLE V

A mutant designated GX8315 was constructed using three single-point mutations. The three point mutations were all found by screening randomly mutagenized subtilisin as described above in Example II. The three mutations were Asn218--->Ser; Gly131--->Asp; and Thr254--->Ala. Oligonucleotide mutagenesis (ODM) was used to make these three changes in wild-type subtilisin.

Figure 8 shows the effect on thermal stability of some combinations of these mutations. The various proteins were incubated at 70°C in 10 mM $CaCl_2$, 50 mM Tris-HCl, 50 mM NaCl at pH 8.0. Aliquots were removed at various times and tested for residual enzymatic activity. Under these conditions, wild-type subtilisin loses half its activity every 19.7 minutes. GX7160 is the same as GX7150 but made by ODM.

Mutant GX7160 (Asn218--->Ser constructed by mutagenesis) loses half its activity every 71.1 minutes, which is 3.59 times as long as the half-time of wild-type.

Mutant GX7169 (Asn218--->Ser and Gly131--->Asp) loses half its activity every 127.7 minutes, which is 6.45 times as long as the half-time of wild-type.

Mutant GX8315 (Asn218--->Ser, Gly131--->Asp, and Thr254 --->Ala) loses half its activity every 230.3 minutes, which is 11.64 times as long as the half-time of wild-type.

EXAMPLE VI

Differential scanning calorimetry and rate of thermal inactivation, as described in Example IV, were performed on three variants:

7150 ASN 218 ---> SER
7142 GLY 131 ---> ASP
7169 ASN 218 ---> SER
GLY 131 ---> ASP

The results are shown in Table II.

TABLE II

Variants of Subtilisin BPN' That Have Enhanced Stability

Variant	ΔT_m (°C)[a]		Rate of Thermal Inactivation[b]	
	10 mM EDTA	10 mM $CaCl_2$	1 mM EDTA	10 mM $CaCl_2$
7150	3.9	2.4	2.6	3.8+0.4
7142	---	0.6	1.0	1.5
7169	3.4	2.9	2.2	6.2

a ΔT_m is the increase in the T_m as measured in a differential scanning calorimetry experiment. The results for two extremes of free calcium concentration, 10 mM EDTA and 10 mM $CaCl_2$, are shown.

b The rates of thermal inactivation are given as multiples of that for the wild type enzyme. The results in 1 mM EDTA were obtained at 45°C, while those in 10 mM $CaCl_2$ were obtained at 65°C.

These data demonstrate that the effect of single mutations on the free energy of unfolding (as measured by T_m) is approximately additive when different single mutations are combined in a single molecule whereas the effect on the rate of inactivation is multiplicative.

EXAMPLE VII

A mutant designated GX7164 was made by oligonucleotide-directed mutagenesis with aspartic acid at location 218. This was done because substitution of serine for asparagine at location 218 had been shown to stabilize subtilisin BPN'. Subtilisin 7164 has a half-life for thermal inactivation which is 1.9 times as large as wild-type subtilisin.

ANNEX M3

International Application No: PCT/US87/00348

MICROORGANISMS

Cotinued Sheet in connection with the microorganism referred to on page 25, line 17 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [5]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit [6]	Accession Number [7]
06 February 1986	ATCC 53459

B. ADDITIONAL INDICATIONS [8] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

Bacillus subtilis, GX 7150

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [9] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [10] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [11] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer) Annette Short

☐ The date of receipt (from the applicant) by the International Bureau [12]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

(January 1985)

WHAT IS CLAIMED IS:

1. A method for mutating cloned DNA and thereafter identifying mutants comprising:

(a) creating a single-stranded target region in a cloned DNA segment;

(b) introducing a mutation into the single-stranded target region by treating the target region with a chemical or biological mutagenizing agent capable of introducing mutations into single-stranded DNA;

(c) rendering the target region double-stranded to form mutated double-stranded DNA;

(d) transforming a microorganism with the mutated double-stranded DNA present in an expression vector;

(e) cultivating the transformed microorganism under conditions wherein the mutated DNA is expressed to form an expression products; and

(f) screening the expression products to identify a desired mutation in the DNA segment.

2. The method of claim 1 wherein said cloned DNA codes for an enzyme and the expression product is a variant of said enzyme.

3. The method of claim 2 wherein the enzyme is subtilisin.

4. The method of claim 2 wherein the enzyme is a serine protease homologous to subtilisin.

5. The method of claim 3 or 4 wherein said

desired mutation provides a subtilisin material of enhanced thermal stability.

6. The method of claim 1 wherein the chemical mutagenizing agent is selected from the group consisting of sodium bisulfite, nitrous acid and formic acid.

7. The method of claim 1 wherein the microorganism is B. subtilis.

8. The method of claim 1 wherein the transformed microorganism is cultivated in the presence of a filter material to which said expression product binds under conditions of cultivation wherein the expression product is transferred to and bound to the filter material; and wherein the screening step includes treating the filter material with expression product bound thereto by subjecting the filter-bound expression product to conditions which would inactivate a wild-type expression product and thereafter contacting the treated expression product with a substrate of said enzyme to identify an expression product of enhanced stability and thereby identify said desired mutation.

9. A cloned DNA segment mutated and identified according to the method of claim 1.

10. A cloned mutant subtilisin gene coding for a subtilisin material which is capable of retaining subtilisin activity at a temperature at which a wild-type subtilisin is inactivated.

11. A cloned mutant subtilisin gene coding for a subtilisin material having another amino acid substituted for asparagine at amino acid position 218 of subtilisin.

12. The gene of claim 11 coding for subtilisin material having serine or asparatic acid substituted for asparagine at amino acid position 218 of subtilisin.

13. The gene of claim 11 having at least one additional amino acid substitution.

14. A cloned mutant subtilisin gene coding for a mutant subtilisin material having serine substituted for asparagine at amino acid position 218 and aspartic acid substituted for glycine at amino acid position 131 of subtilisin.

15. A cloned mutant subtilisin gene coding for a mutant subtilisin material having serine substituted for asparagine at amino acid position 218 and alanine substituted for threonine at amino acid position 254 of subtilisin.

16. A cloned mutant subtilisin gene coding for a mutant subtilisin material having serine substituted for asparagine at amino acid position 218 and serine substituted for glycine at amino acid position 166.

17. A cloned mutant subtilisin gene coding for a mutant subtilisin material having serine

substituted for asparagine at amino acid position 218 and aspartic acid substituted for glycine at amino acid position 131 and alanine substituted for threonine at amino acid position 254 of subtilisin.

18. A cloned mutant subtilisin gene coding for a mutant subtilisin material having aspartic acid substituted for glycine at amino acid position 131 of subtilisin.

19. A cloned mutant subtilisin gene coding for a mutant subtilisin material having proline substituted for serine at amino acid position 188 of subtilisin.

20. A cloned mutant subtilisin gene coding for a mutant subtilisin material having glutamic acid substituted for alanine at amino acid position 116.

21. A cloned mutant subtilisin gene coding for a mutant subtilisin material having isoleucine substituted for leucine at amino acid position 126 of subtilisin.

22. A cloned mutant subtilisin gene coding for a mutant subtilisin material having threonine substituted for serine at amino acid position 53 of subtilisin.

23. The cloned mutant subtilisin gene of claim 10 present in B. subtilis strain GX7150 (ATCC No. 53459).

24. A method for producing and identifying a subtilisin material of enhanced thermal stability comprising:

(a) treating a cloned subtilisin gene, at least a portion of which is in single-stranded form, with a mutagenizing agent selected from the group consisting of sodium bisulfite, nitrous acid, and formic acid to introduce at least one mutation into said gene;

(b) rendering the treated gene double-stranded;

(c) transforming B. subtilis with the treated double-stranded gene present in an expression vector;

(d) cultivating the transformed B. subtilis in the presence of a filter material to which subtilisin is capable of binding, under conditions wherein the treated gene is expressed to form an expression product and the expression product is transferred to and bound to the filter material;

(e) incubating the filter material to which the expression product is bound at a temperature at or above a temperature at which wild-type subtilisin is inactivated; and

(f) contacting the incubated expression product with a subtilisin substrate material to identify a subtilisin material of enhanced thermal stability, and thereby identify a mutant subtilisin gene coding for a subtilisin material of enhanced thermal stability.

25. A mutant subtilisin material which is capable of retaining subtilisin activity at a

temperature at which wild-type subtilisin is inactivated.

26. The mutant subtilisin material of claim 25 wherein said material is mutated subtilisin.

27. The mutant subtilisin material of claim 25 wherein said material is a mutated homologous serine protease to subtilisin.

28. The mutant subtilisin material of claim 25 produced according to the method of claim 1.

29. A mutant subtilisin material containing an amino acid substitution or any combination of amino acid substitutions which renders said mutant subtilisin material capable of retaining subtilisin activity at a temperature at which wild-type subtilisin is inactivated, produced according to the method of claim 1.

30. A mutant subtilisin material having any amino acid substitution which corresponds to the position of an amino acid substitution in a first mutant subtilisin material which renders said first mutant subtilisin material capable of retaining subtilisin activity at a temperature at which wild-type subtilisin is inactivated.

31. A mutant subtilisin material having another amino acid substituted for asparagine at amino acid position 218 of subtilisin.

32. The mutant subtilisin material of claim 31 having serine or asparatic acid substituted for asparagine at amino acid position 218 of subtilisin.

33. The mutant subtilisin material of claim 31 having at least one additional amino acid substitution.

34. A mutant subtilisin material having serine substituted for asparagine at amino acid position 218 and aspartic acid substituted for glycine at amino acid position 131 of subtilisin.

35. A mutant subtilisin material having serine substituted for asparagine at amino acid position 218 and alanine substituted for threonine at amino acid position 254 of subtilisin.

36. A mutant subtilisin material having serine substituted for asparagine at amino acid position 218 and serine substituted for glycine at amino acid position 166.

37. A mutant subtilisin material having serine substituted for asparagine at amino acid position 218 and aspartic acid substituted for glycine at amino acid position 131 and alanine substituted for threonine at amino acid position 254 of subtilisin.

38. A mutant subtilisin material having aspartic acid substituted for glycine at amino acid position 131 of subtilisin.

39. A mutant subtilisin material having proline substituted for serine at amino acid position 188 of subtilisin.

40. A mutant subtilisin material having glutamic acid substituted for alanine at amino acid position 116.

41. A mutant subtilisin material having isoleucine substituted for leucine at amino acid position 126 of subtilisin.

42. A mutatnt subtilisin material having threonine substituted for serine at amino acid position 53 of subtilisin.

43. The mutant subtilisin material of claim 15 produced by cultivating B. subtilis strain GX7150 (ATCC No. 53459).

44. A method for producing the mutant subtilisin material of claim 43, comprising cultivating B. subtilis strain GX7150 (ATCC No. 53459) under protein-producing conditions.

45. A B. subtilis strain carrying the mutant gene of claim 10.

46. A B. subtilis strain carrying the mutant gene of claim 11.

47. B. subtilis strain GX7150 (ATCC No. 53459).

48. A thermally stable mutant subtilisin serine protease material obtained by the following steps:

(a) mutating a first subtilisin by the method of claim 1 and identifying any thermally stabilizing amino acid mutations;

(b) obtaining the amino acid sequence of a second naturally-occurring subtilisin;

(c) aligning the amino acid sequences of said first mutated subtilisin with the amino acid sequence of second naturally-occurring subtilisin;

(d) deleting those amino acid mutations of the first mutated subtilisin which fall at or next to gaps in the amino acid sequences of said first and second subtilisins;

(e) mutating said second subtilisin by oligonucleotide-directed mutagenesis to match the mutations of said first mutated subtilisin; and

(f) producing a thermally stable mutant subtilisin material.

49. A method for improving the removal of proteinaceous stains on fabric comprising adding a mutant subtilisin material of any of claims 30-43 or 48 to a washing preparation and cleaning said stained fabric with said washing preparation.

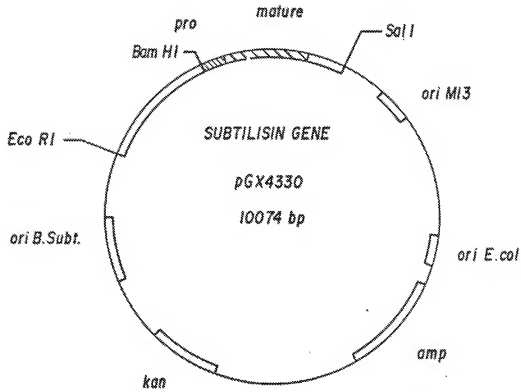

pro
mature
Bam HI
Sal I
ori MI3
Eco RI
SUBTILISIN GENE
pGX4330
10074 bp
ori B.Subt.
ori E.coli
amp
kan

FIG. 1

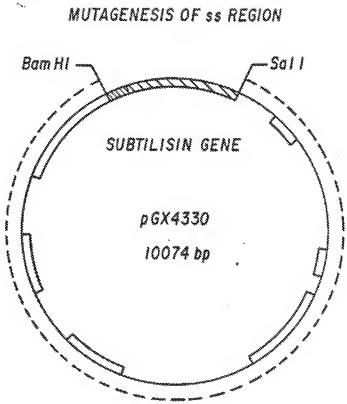

MUTAGENESIS OF ss REGION
Bam HI
Sal I
SUBTILISIN GENE
pGX4330
10074 bp

FIG. 2

Ser
G

TAC GGG GCG TAC AAC GGT ACG TCA AGT
Tyr Gly Ala Tyr Asn Gly Thr Ser Met
215 220

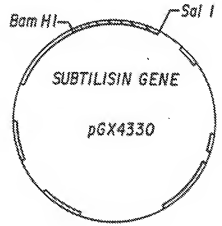


FIG. 3

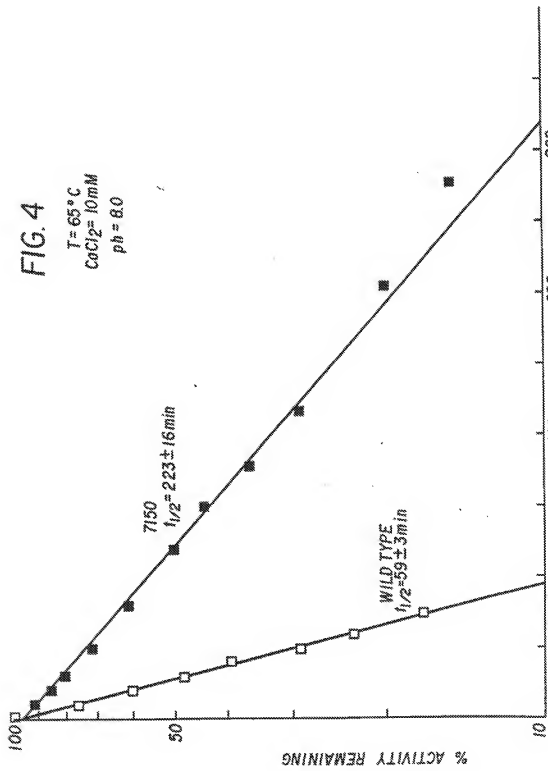
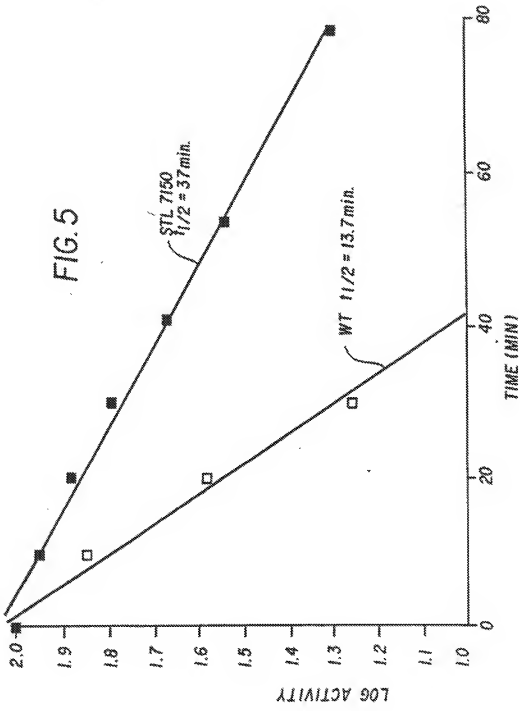


FIG. 4
T = 65°C
CaCl2 = 10mM
ph = 8.0
7150
t1/2 = 223 ± 16min
WILD TYPE
t1/2 = 59 ± 3min
% ACTIVITY REMAINING
100
50
10
0
200
400
600
800
TIME (MIN)

FIG. 5


LOG ACTIVITY
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0
20
40
60
80
TIME (MIN)
STL 7150
t1/2 = 37min.
WT t1/2 = 13.7min.

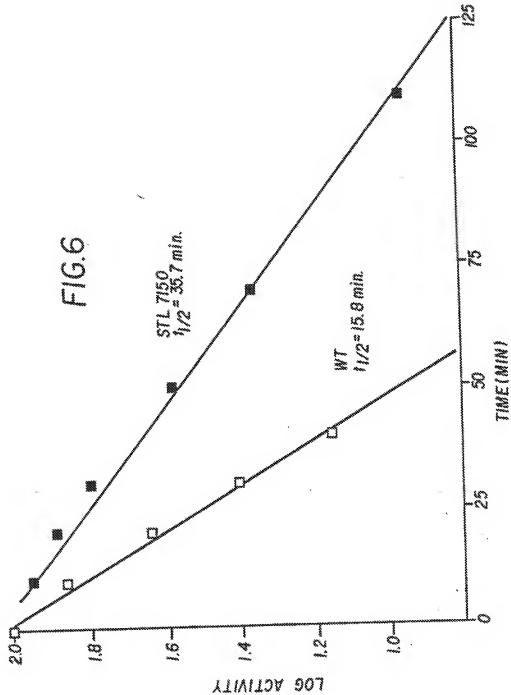


FIG.6
STL 7150
t1/2 = 36.7 min.
WT
t1/2 = 15.8 min.
LOG ACTIVITY
2.0
1.8
1.6
1.4
1.2
1.0
0
25
50
75
100
125
TIME (MIN)

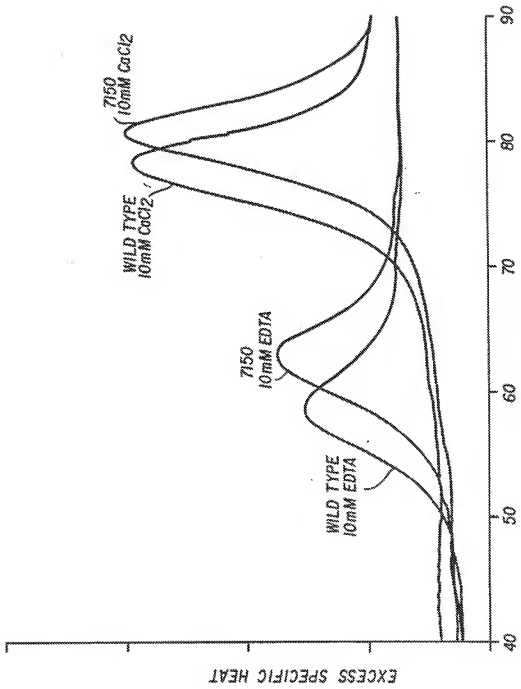

7150
10mM CaCl2
WILD TYPE
10mM CaCl2
7150
10mM EDTA
WILD TYPE
10mM EDTA
EXCESS SPECIFIC HEAT
40
50
60
70
80
90
TEMPERATURE (°C)

FIG. 7

LOG ACTIVITY %
2.
1.5
1.
.5
0.
0
100
200
300
400
WILD TYPE
GX7130
Asn 218 — Ser
GX7150
Asn 218 — Ser & Gly 131 — Asp
GX7169
GX8315
Asn 218 — Ser; Gly 131 — Asp; Thr 254 — Ala
GX7130 WILD TYPE
T(1/2) = 19.7
Mu / Wty = 1.00
GX7150
T(1/2) = 71.1
Mu / Wty = 3.59
GX7169
T(1/2) = 127.7
Mu / Wty = 6.45
GX8315
T(1/2) = 230.3
Mu / Wty = 11.64

FIG. 8

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00348

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL[4]: C12Q 1/68;C12P 21/00;C12N 15/00,9/56,1/20;D06M 16/00; C07H 21/00
U.S. CL : 435/6.68,172.3,222,253,263; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/6,68,172.1,172.3,222,253,263,264,839 935/78,14 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

COMPUTER SEARCH: BIOSIS 1977-1987; CHEMICAL ABSTRACTS 1967-1987 LEXPAT

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [15]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	Nucleic Acids Research Vol. 13, No.24, issued 20 December 1985 (Oxford, England), M. Jacobs et al, "Cloning, sequencing and expression of subtilisin Carlsberg from Bacillus licheniformis" see page 8913.	1-49
Y	Chemical Abstracts, Vol. 103, No. 5, issued 05 August 1985 (Columbus, OH, USA), D.A. Estell et al, "Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation", see page 115, column 1, the abstract No. 33007f, J. Biol. Chem. 1985 260(11), 6518-21 (Eng).	1-49

* Special categories of cited documents: [12]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
03 April 1987	28 APR 1987
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Jeremy M. Jay

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

P,Y	Chemical Abstracts, Vol. 104, No. 19, issued 12 May 1986 (Columbus, OH, USA), D.A. Estell et al, "Site-directed mutagenesis of the active site of subtilisin BPN'", see page 130, column 1, the abstract No. 162674r, World Biotech, Rep. 1984, 2, 181-7 (Eng.)	1-49

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No **
Y	Chemical Abstracts, Vol. 102, No. 23, issued 10 June 1985 (Columbus, OH, USA), N. Vasantha et al, "Cloning of a serine protease gene from Bacillus amyloliquefaciens and its expression in Bacillus subtilis", see page 160, column 1, the abstract No. 198918w, Genet. Biotechnol. Bacilli 1983, 2, 163-72 (Eng.)	1-49
Y	Chemical Abstracts, Vol. 102, No. 23, issued 10 June 1985 (Columbus, OH, USA), J.A. Wells et al, "Cloning and sequencing of a region controlling efficient expression of subtilisin from Bacillus amyloliquefaciens", see page 160, column 1, the abstract No. 198919x, Genet. Biotechnol. Bacilli 1983, 2, 173-180 (Eng).	1-49
Y	Proceedings National Academy of Sciences USA, Vol. 83, issued February 1986 (Washington D.C., USA), H. Liao et al, "Isolation of a thermostable enzyme variant by cloning and selection in a thermophile", see page 576	1-49